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Title

Understanding genetic diversity of relict forests. Linking long-term isolation legacies and current habitat fragmentation in *Abies pinsapo* Boiss.

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Abstract

Increasing variability and uncertainty regarding future climate provide new challenges for the conservation of endangered tree species. For example, threat status can be impacted by genetic diversity, where forest trees show reduced geographic range size, isolated populations and fragmented distribution. We place the conservation insights of population genetic structure in a climate change context, using as experimental system a relict drought-sensitive fir (*Abies pinsapo* Boiss.). Nuclear (nSSR, ISSR) and chloroplast (cpSSR) markers were analysed to investigate the extent to that *A. pinsapo* evidences ongoing genetic erosion, isolation and divergent genetic diversity, among populations, elevations and cohorts (young, adult and old trees). We obtained contrasting patterns among chloroplast and nuclear markers. Based on cpSSRs, the highest genetic distances were found in the western portion of the distribution, while based on both nSSRs and ISSRs, differentiation appeared in the eastern portion of the distribution. Evidence for bottlenecks and genetic drift were found in all the studied populations, as well as low among-population genetic differentiation. Land use legacies e.g. impacting current forest structural diversity might be related to observed genetic diversity. No evidence of demographic genetic erosion among cohorts was found. Conservation efforts should focus on reducing the probability of occurrence of stochastic events such as fires and habitat loss due to human impacts or climate change to maximise *A. pinsapo* population sizes. Further research on adaptive potential should focus on identifying active genetic management strategies that might improve adaptation to future climates in such endangered relict species.

Keywords: Gen flow; Genetic diversity; Genetic drift; Inbreeding; Adaptive management; Circum-mediterranean firs; Conservation genetics; Microsatellite marker.

Highlights

- *Abies pinsapo* maintained relatively high genetic diversity.
- *A. pinsapo* shows low among-population genetic differentiation.
- Local climate and land-use legacies were related to haplotype diversity.
- Nuclear and chloroplast markers provide contrasting patterns of genetic diversity.
- Bottlenecks and genetic drift evidences were found in all the studied populations.
- Genetic erosion was not observed among cohorts of saplings, mature and old trees.

1. Introduction

High variability and uncertainty regarding potential future climates provides new challenges for the adaptive management of drought-sensitive forest ecosystems (Jump and Peñuelas 2005; Aitken et al., 2008; Alberto et al., 2013). The threat status of several species relies to some extent on genetic diversity, for example, in forest trees with reduced geographic range size, isolated populations and fragmented distribution (Fady and Conord 2010; Hampe and Jump 2011; Rehm et al., 2015). Tree species, as long-lived and sessile organisms, mainly depend on their current genetic variation to develop locally-adapted phenotypes (Petit and Hampe 2006). Consequently, understanding evolutionary consequences of global climate change and its long-term effects on biodiversity requires the investigation of the effect of range size, geographic isolation and fragmentation on intraspecific genetic diversity (Kuparinen et al., 2010; Franks and Hoffmann 2012; Alberto et al., 2013).

The practical application of the theories underlying adaptive capacity and genetic diversity to the management of relict populations is a main concern regarding conservation biology (Neale and Wheeler 2019). Here, well-known patterns, such as the relationship between heterozygosity and population fitness, support the need to assess the processes of genetic erosion, genetic drift, or inbreeding to conserve genetic diversity (Reed and Frankham 2003). Furthermore, exploration of the limitation of gene flow due to isolation and small population sizes provides the basis to understand the current spatial genetic structure and to define reliable conservation strategies aimed to reduce the loss of genetic diversity (Ledig et al., 1997, 2002; Jaramillo-Correa et al., 2006, 2008; Eliades et al., 2011; Aleksić and Geburek 2013; Awad et al., 2014).

Reduced gene flow and distinct ecotypes are expected in remnant populations (Kremer et al., 2012). Thus, quantification of the genetic diversity among populations of relict species is required to improve conservation planning (Neale and Wheeler 2019). Furthermore, reliable spatial and temporal inference of recent genetic and demographic changes may contribute towards a better understanding of the evolutionary process accounting for sometimes high levels of genetic diversity in relict species confined to climatic refugia (Hampe and Jump 2011). Range size plays here an essential role in shifting patterns of genetic diversity (Hampe and Petit 2005). Specifically, low genetic variation but high genetic differentiation is theoretically expected in relict populations located in fragmented landscapes (Hampe and Jump 2011; Rehm et al., 2015). Notwithstanding, it has been reported that endemic species, usually with restricted geographic ranges, may also show high genetic diversity (Ledig et al., 1997, 2002; Eliades et al., 2011; Aleksić and Geburek 2013).

Biogeography has an important effect on genetic diversity and structure. For instance, the conifers from the Mediterranean basin show higher genetic diversity, compared to those from other regions (Fady-Welteren,

2005). Furthermore, decreasing genetic diversity has also been recognized within populations of several taxa in the western locations across the Mediterranean Basin (Fady and Conord 2010). Hence, Mediterranean relict forests provide suitable experimental systems to investigate the effect of range size, geographic isolation and fragmentation in shaping patterns of genetic diversity (Hampe and Jump 2011). These biogeographic and evolutionary characteristics enhance the need to design adaptive management guidelines aimed to preserve Mediterranean relict forests (Hampe and Petit 2005; Hampe and Jump 2011; Rehm et al., 2015).

Among them, some taxa seem to be particularly vulnerable to the current changing climate, as might be the case of the relict circum-mediterranean firs (*Abies* Mill.), tree species that are, in many cases, near to their tolerance limits, and therefore might be considered among the most sensitive ecosystems to current climate change (Sánchez-Salguero et al., 2017). The current genetic diversity of the circum-mediterranean firs has been recognized to a large degree as a consequence of ice age isolation in southern refugia and postglacial colonization northwards (Linares, 2011), while these phylogeographical patterns may be currently constraining the adaptive capacity of those remnant fir forests (Fady and Conord 2010; Liepelt et al., 2010). Furthermore, land-use changes that have occurred during the last decades represent an additional predisposing factor to climate-induced decline and mortality for several Mediterranean fir forests (Linares et al., 2009; Lechuga et al., 2017, 2019; Alba-Sánchez et al., 2019).

Here we place the conservation insights of population genetic structure in a climate change context, using as an experimental system the relict drought-sensitive fir *Abies pinsapo* Boiss. To date, it is not known whether *A. pinsapo* lost genetic diversity following Holocene climate change, what the limits to gene flow might be, or whether inbreeding and reduced gene pool are currently constraining its range of adaptation. We investigate the genetic structure patterns at different spatial scales, as well as accounting for a demographic, time-related, perspective by sampling young, adult and old trees of this species. We hypothesise low genetic diversity for the studied *A pinsapo* forests, according to their relict features, as compared to other non-relict circum-Mediterranean firs. A second hypothesis is that *A. pinsapo* should show evidence of inbreeding and recent bottlenecks due to long-term isolation of its remnant populations. Consequently, geographic location is hypothesized here to affect genetic diversity, both within and among *A. pinsapo* populations. We seek to provide information to guide management policies aiming to reduce future extinction risk for this endangered tree.

2. Materials and Methods

2.1. Abies pinsapo as experimental model

We focus on *Abies pinsapo* Boiss., a Tertiary relict tree species, endemic of the Baetic Range (Southern Spain), closely related to North-Moroccan populations *A. marocana* Trab., and *A. tazaotana* Villar., (Terrab et al., 2007; Jaramillo-Correa et al., 2010; Dering et al., 2014; Sanchez-Robles et al., 2014). Currently, *A. pinsapo* is listed as endangered, as well as other circum-mediterranean firs, by the International Union for Conservation of Nature (Arista et al., 2011). This species represents the southernmost European fir species (Sánchez-Salguero et al., 2017). Likewise, as a climate relict, *A. pinsapo* displays limited possibilities of migration, while its long-term persistence has been related to climatic refugia (Alba-Sánchez et al., 2010; Linares 2011; Alba-Sánchez et al., 2019). Currently, *A. pinsapo* populations are mainly located on north-facing slopes between 1000 and 1800 m.a.s.l. (Linares et al., 2009). Fragmented populations of *A. pinsapo* experienced an expansion and densification from scattered remaining stands following the implementation of conservation measures in the middle of the 20th Century (Linares et al., 2009; Lechuga et al., 2017). However, recent climate change has been related to increasing drought and *A. pinsapo* forest decline (Linares et al., 2009, 2011; Lechuga et al., 2017, 2019). Recurrent mortality events, mainly at low-elevation sites within the elevation range of *A. pinsapo* have been noted, whilst the high-elevation sites yielded a recent growth enhancement and increasing forest cover (Linares et al., 2009; Lechuga et al., 2019). Given the limited migration potential of this climate relict, climate change may be imposing an enhanced threat for the conservation of this species (Hampe and Petit 2005; Kuparinen et al., 2010; Hampe and Jump 2011; Rehm et al., 2015).

2.2. Sampling Design and Plant Material

A. pinsapo forests are mainly restricted to two locations in the Baetic Range, each subjected to a conservation designation (Figure 1): the National Park Sierra de las Nieves (36°41′53″N, 4°59′50″W), accounting for about 5800 ha, and the Biosphere Reserve Sierra de Grazalema (36°46′25″N, 5°24′35″W), accounting for about 2000 ha. Although, scattered stands and isolated trees, assumed to represent the remains of former larger populations, are rather common throughout the current *A. pinsapo* range (Figure 1). Sampling was performed in these main ranges, by selecting individuals through several altitudinal gradients, between 1100 and 1700 m a.s.l., thereafter grouped as low-, middle- and high-elevation (Table 1), including some lower and upper altitudinal ecotones (Linares et al., 2009). We also studied the genetic structure of trees belonging to different cohorts by focusing on three watersheds (Saucillo, Caucon and Animas), located in Sierra de las

Nieves National Park, which represents the main area covered by dense *A. pinsapo* forests (Alba-Sánchez et al., 2019).

Here, based on previous dendrochronological research (Linares et al., 2009, 2011), we sampled individuals belonging to different ages in order to test whether the genetic structure of different cohorts is demographically stable or might be undergoing genetic erosion (Wehenkel and Saenz-Romero 2012). We sampled needles of old individuals selected at distance intervals of about 50 m, and their closest mature tree and juvenile sapling. Mean ages were 138±36 years in old trees, 65±9 years in mature trees, and 26±11 years in juvenile trees, respectively. A total of 202 trees were sampled and the collected needles stored at −80°C prior to DNA extraction.

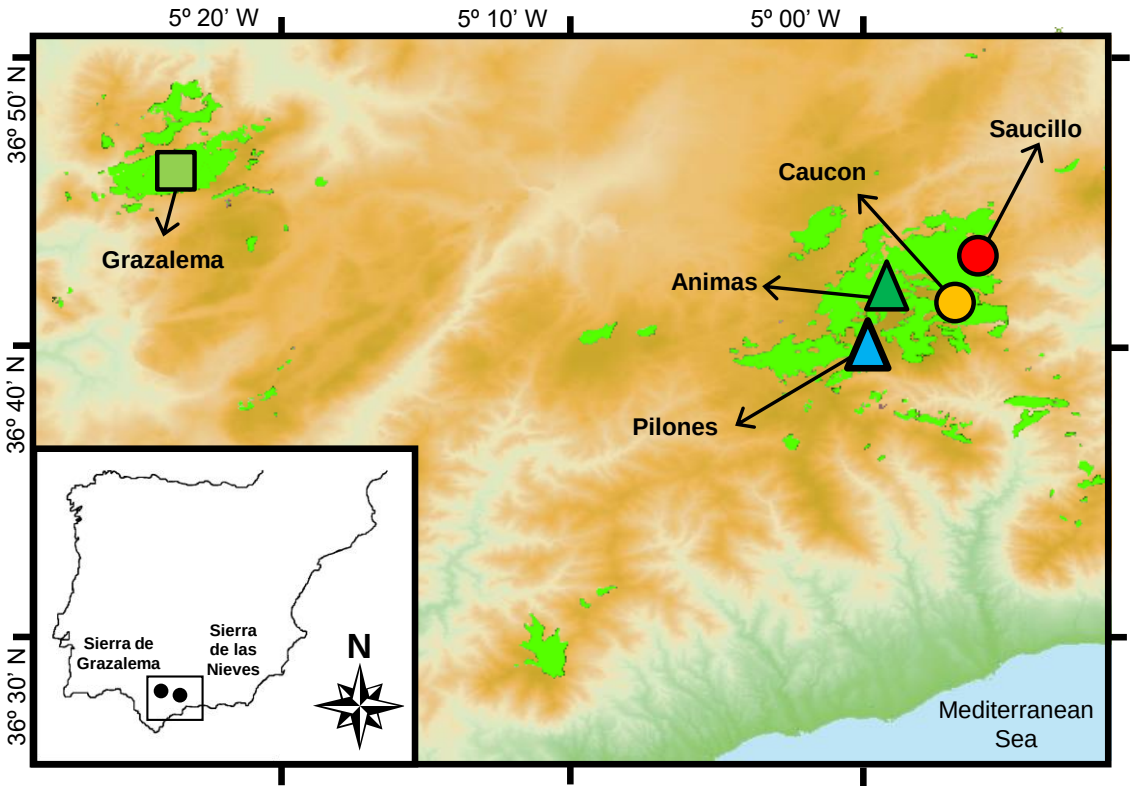


Figure 1. Study area (left bottom inset), distribution of *Abies pinsapo* forests (green shape) and sampling location (symbols) within the mountain ranges of the Sierra de las Nieves National Park and the Sierra de Grazalema Biosphere Reserve. See also Table 1.

153 *2.3. DNA Extraction, Microsatellite and Inter-Microsatellite Genotyping.*

154 We studied three different genomic microsatellites (simple sequence repeats; SSR) as neutral molecular
155 markers with different inheritances: nuclear markers (nuclear microsatellites, nSSR, and inter-microsatellites,
156 ISSR), which are biparentally inherited; and chloroplast markers (chloroplast microsatellites, cpSSR),
157 paternally inherited in conifers (Liepelt et al., 2002; Petit et al., 2005; Neale and Wheeler 2019). Total genomic
158 DNA was extracted and purified according to QIAGEN DNeasy plant mini kit protocol (Pérez-González et al.,
159 2018). We used 8 nuclear microsatellites to perform genomic DNA amplification: NFF2, NFF3, NFH15, NFH3
160 and NFF7 developed for *A. nordmanniana* Stev. (Hansen et al., 2005); and Pin8, Pin20 and Pin48 developed for
161 *A. pinsapo* (Sánchez-Robles et al., 2012). In addition, we amplified 3 chloroplast microsatellites: Pt30204,
162 Pt71936 and Pt15169 developed for *Pinus thunbergii* (Vendramin et al., 1996). Microsatellite selection was
163 done based on previous studies that prove that they yield enough polymorphic bands in other species
164 phylogenetically related with *A. pinsapo*. 19 ISSR primers from the University of British Columbia, Canada
165 (UBC) were tested in two individuals to select those that yield more polymorphic bands (see the detailed
166 methodology in the Electronic Supplementary Material, Appendix 1).
167

168 **Table 1.** Main characteristics and sample size of the studied *Abies pinsapo* populations. The number
169 of young (Y), Adult (A), and old (O) trees is indicated between parenthesis.

Population	Site (Code)	Elevation classes	Latitude (N)	Longitude (E)	Elevation (m a.s.l.)	N (age classes)
Saucillo	S	Low (SL)	36º 42' 43" - 36º 43' 33"	4º 57' 55"- 4º 59' 16"	1178-1259	16 (Y=6, A=6, O=4)
		Middle (SM)			1295-1340	16 (Y=5, A=6, O=5)
		High (SH)			1450-1521	14 (Y=5, A=4, O=5)
Caucon	C	Low (CL)	36º 42' 15" - 36º 42' 45"	4º 57' 50" - 4º 58' 28"	1112-1190	21 (Y=7, A=7, O=7)
		Middle (CM)			1225-1289	39 (Y=13, A=13, O=13)
		High (CH)			1307-1399	15 (Y=5, A=5, O=5)
Animas	A		36º 41' 46" - 36º 41' 56"	5º 00' 59" - 5º 01' 04"	1589-1684	50 (Y=20, A=10, O=20)
Grazalema	G	Low (GL)	36º 45' 53" - 36º 46' 28"	5º 24' 8" - 5º 25' 39"	1165-1287	6
		Middle (GM)			1305-1379	6
		High (GH)			1391-1479	6
Pilones	P		36º 41' 36"	5º 01' 09"	1716-1740	13

170

171 *2.4. Statistical Analysis*

172 *2.4.1. Hardy-Weinberg equilibrium and null alleles*

173 Neutrality among the molecular markers was tested by scanning the nSSR dataset for loci under
174 differential selection, using outlier loci analysis in BayeScan 2.01 (Foll and Gaggiotti 2008). We examined the
175 presence and frequency of null alleles using the Expectation Maximization (EM) algorithm in FreeNA (Chapuis
176 and Estoup 2007). Null alleles frequencies were estimated for each locus, as well as for the mean frequency of
177 null alleles in each population. Since the presence of null alleles may overestimate the population genetic
178 differentiation, an Fst statistic was computed excluding null alleles (ENA) and without the ENA correction
179 method. Simulation studies suggest that null alleles with frequencies between 5% and 8% should have only
180 minor effects on estimates of population differentiation (Chapuis and Estoup 2007). We used bootstrapping to
181 estimate 95% confidence intervals, running 50000 replicates per locus.

182 We calculated allele frequencies for each polymorphic locus obtained by ISSR assuming Hardy-Weinberg
183 equilibrium. GeneAlExv6.502 was used to estimate the frequency of the null alleles (q) by taking the square
184 root of the frequency of the null homozygotes (absence of a band). Then, we obtained the frequency of the
185 dominant allele as $p=1-q$. We removed bands with frequencies higher than $1-(3/N)$, where N is the population
186 sample size, to avoid ISSR underestimates of genetic variation (Chapuis and Estoup 2007).

187 *2.4.2. Within-populations and within-cohorts genetic diversity*

188 Since the nuclear (nSSR and ISSR) and chloroplast (cpSSR) neutral molecular markers used in this study
189 have different characteristics (diploid codominant, diploid dominant and haploid, respectively), we estimated
190 different parameters with each one to describe the neutral genetic diversity of the species, within different
191 populations and within different cohorts. For all neutral markers, we calculated the following parameters:
192 Percentage of polymorphic loci (PPL), number of private alleles (NPA) and number of effective alleles (Ne).
193 For nSSR and ISSR, expected heterozygosity (He) was also estimated. For nSSR we calculated observed
194 heterozygosity (Ho); and Wright's fixation indices for within-subpopulation to test the inbreeding index (FIS)
195 (Weir and Cockerham 1984). We calculated unbiased diversity (h) and haplotype frequencies based on cpSSR
196 markers. All these analyses were carried out with GeneAlEx 6.501 (Peakall and Smouse 2006, 2012).

197 Finally, rarefied allelic richness (Ar) was estimated based on nSSR molecular markers with FSTAT, as
198 well as number of migrants (Nm) among populations and spatial and temporal cohorts. In addition, a Student t
199 test was implemented with statistical significance al the 5% nominal level of the difference between the mean
200 value of Ho and He across all samples for all nSSR loci in order to test again a possible effect of selection. The

201 statistically significance of the differences of these parameters among spatial and temporal cohorts were
202 estimated by means of an unpaired Student t test for unequal variances.

203 To detect any recent severe reduction in effective population size or possible expansion events in *A.*
204 *pinsapo* populations, BOTTLENECK 1.2.02 was used on the nSSR dataset (Cornuet and Luikart 1996; Luikart
205 et al., 1998; Piry et al., 1999; Petit et al., 2005). Bottlenecks cause low-frequency alleles to become transitorily
206 less abundant (<0.1), while more intermediate-frequency alleles increase (Luikart et al., 1998). BOTTLENECK
207 correlates expected heterozygosity (He) with observed heterozygosity (Ho) at mutation-drift equilibrium. The
208 two-phased model (TPM) of mutation was applied as the most appropriate for microsatellite data (Piry et al.,
209 1999). For TPM, we used 5% and 15% of multistep changes (Probability 95% and 85 %, respectively) and a
210 variance among multiple (12) steps (Piry et al., 1999). For each population, 2000 simulations were performed in
211 all datasets. Significance was assessed using the implemented Wilcoxon sign-rank test, which determines
212 whether or not the average of standardized differences between Ho and He is significantly different from zero
213 (Cornuet and Luikart 1996). Significant heterozygote excess relative to the number of alleles indicates a recent
214 population bottleneck.
215
216 *2.4.3. Among-populations and among-cohorts genetic differentiation*
217 We summarized genetic differentiation among the different populations of *A. pinsapo* using pairwise Fst
218 and pairwise Nei‘s standard genetic distances on all neutral markers. Spatial limitation of gene flow resulting in
219 an isolation by distance pattern was analysed by a Mantel-Test, performed with GeneAlEx version 6.501, using
220 genetic and geographical distances (Peakall and Smouse 2006; Peakall and Smouse 2012). Significance was
221 estimated via 9999 permutations. We estimated partitioning of genetic variation among locations, elevations
222 and age cohorts, as well as within populations, using a hierarchical analysis of molecular variance (AMOVA) in
223 GenAlex (Peakall and Smouse 2006; 2012). For nSSR markers, the analysis was based on allele identity and
224 allele size by using Fst and Rst respectively. For ISSR and cpSSR markers, the analysis used PhiPT, a measure
225 that allows the suppression of intra-individual variation, comparing dominant and haploid markers (Peakall and
226 Smouse 2006). 9999 random permutations were carried out for significance testing in all cases. To establish the
227 effects of geographic, cohort and altitudinal distances on genetic differentiation, we applied a multivariate
228 principal coordinates analysis (PCoA) based on pairwise Nei’s standard genetic distances among populations in
229 GenAlEx (Peakall and Smouse 2006).
230 To estimate the optimum number of subpopulations (K), we applied a model-based clustering algorithm in
231 a Bayesian framework and the Markov chain Monte Carlo (MCMC) algorithm with STRUCTURE (ST,

thereafter; Earl and vonHoldt 2012) under the assumption that each cluster is in optimal H-W equilibrium and linkage equilibrium (LE). ST analysis used correlated allele frequencies and the admixture model, which allowed for mixed recent ancestries of individuals and assigned the proportion of the genome of each individual to the inferred clusters without prior population information. We ran the analysis for K values of 1-10 with 10 independent runs each and a burn-in period of 100000 and thereafter 200000 MCMC, without the inclusion of geographic coordinates. The number of genetically homogeneous clusters (K) was identified by following the method developed by Evanno et al. (2005). The results were summarized in ST HARVESTER (Earl and vonHoldt 2012). ST analysis calculated the membership coefficient of individuals (individuals Q-matrix) for each of the defined genetic clusters and the proportion of ancestry of each population in each cluster (population Q-matrix) by averaging the membership coefficient of all individuals in a population. The populations were assigned to a specific cluster based on an arbitrary threshold of $Q > 0.80$ regardless of the values of the rest of the clusters.

2.5. Demographic history

The software DIYABC v2.1.0 was used to infer past demography of the studied populations (Cornuet et al., 2014). Both nuclear (nSSR) and chloroplast (cpSSR) neutral markers were used to perform the analysis (Supplementary material, Appendix 2). The same prior parameters were defined for all scenarios. The default values of the priors were used for all parameters. Minimum estimate of generation time of 20 years was used in the calculation of number of generations, since *A. pinsapo* trees start to produce seeds at this age (Authors' personal observation; Arista and Talavera 1994a). Summary statistics obtained by run one million simulations included mean number of alleles, mean genetic diversity, mean size variance across loci and mean Garza-Williamson's M index across loci for each population and for population pairs, Fst, classification index, shared allele distance and du2 distance were also included. The 10% simulated data sets closest to observed data set was used to estimate posterior distributions of parameters through a local linear regression procedure. Seven evolutionary scenarios were tested with DIYABC 2.1.0 based on the results obtained here and in previous studies (Dering et al., 2014; Sanchez-Robles et al., 2014) to test genetic differentiation among populations and assuming hypothetical divergence times (Electronic Supplementary Material, Appendix 2). Models were compared by estimating their posterior probabilities using the direct estimation and logistic regression methods (Cornuet et al., 2014).

3. Results

3.1. Hardy-Weinberg equilibrium (HWE) and null alleles

The W parameter estimated for nSSR markers did not show any locus under differential selection according to any of the applied criteria (all samples together, each population separately and different cohorts, both temporal and spatial). In addition, mean observed heterozygosity (Ho = 0.528±0.031) was not significantly different from the mean expected heterozygosity under HWE (He = 0.596±0.034; Student t test, P=0.095; Table 2). Likewise, FreeNA analysis did not show significant evidence for null alleles, since the estimated null allele frequency using the EM algorithm was 5.1 %; variation in Fst estimation was negligible after excluding null alleles (ENA Fst=0.0602), compared to Fst without ENA correction (Fst = 0.0658). EM algorithm did not show evidence of high frequencies of null alleles for any of the nSSR loci (Electronic Supplementary Material, Table S1). For ISSR markers, 7 out of the 19 tested oligos generated polymorphic and reproducible bands: 807, 807b18up, 825, 835b10lw, 855, otrob4lw and otrob16lw (University of British Columbia, Canada), which were used to carry out further analysis.

3.2. Genetic diversity of A. pinsapo in Iberian Peninsula

The studied neutral molecular markers yielded a high percentage of polymorphic loci (PPL) (nSSR, PPL=95.83% and cpSSR, PPL=83.33%) with the exception of ISSR which yielded only a 14.81%, indicating that are less effective markers to test the genetic diversity of this species. Neutral genetic diversity of *A. pinsapo* in the Iberian Peninsula was moderately high using all analyzed molecular markers with the exception of ISSR. Thus, only polymorphic loci were included in the subsequent analyses. Particularly, for nSSR, mean rarefied allelic richness (Ar) reached a value of 2.78 for a standarized sample size of n=6 gene copies (Table 2). The overall mean inbreeding coefficient (Fis = 0.150) was statistically different from zero (P<0.001; Table 2). Effective number of alleles (Ne) was 2.825±0.195, Ho was 0.528 and He, 0.596. For cpSSR markers, diversity (h) showed a value of 0.523±0.077 and Ne was 2.541±0.306. Finally, for ISSR markers, He was 0.035+-0.005, but rose to a value of 0.167±0.02 based on polymorphic loci and Ne was 1.058±0.009, reaching the lowest values of all analysed genetic diversity parameters, as expected considering their particularly low PPL (Table 2).

Table 2. Genetic diversity within population parameters of *A. pinsapo* individuals sorted by population, elevation and age cohorts (See abreviations in Table 1) based on the three studied neutral molecular markers (nSSR, cpSSR, ISSR). N, population size; NPA, number of private alleles; Ne, number of effective alleles; Ar, rarified allelic richness; Ho, observed heterozygosity; He, expected heterozygosity; h, genetic diversity; Fis, inbreeding index.

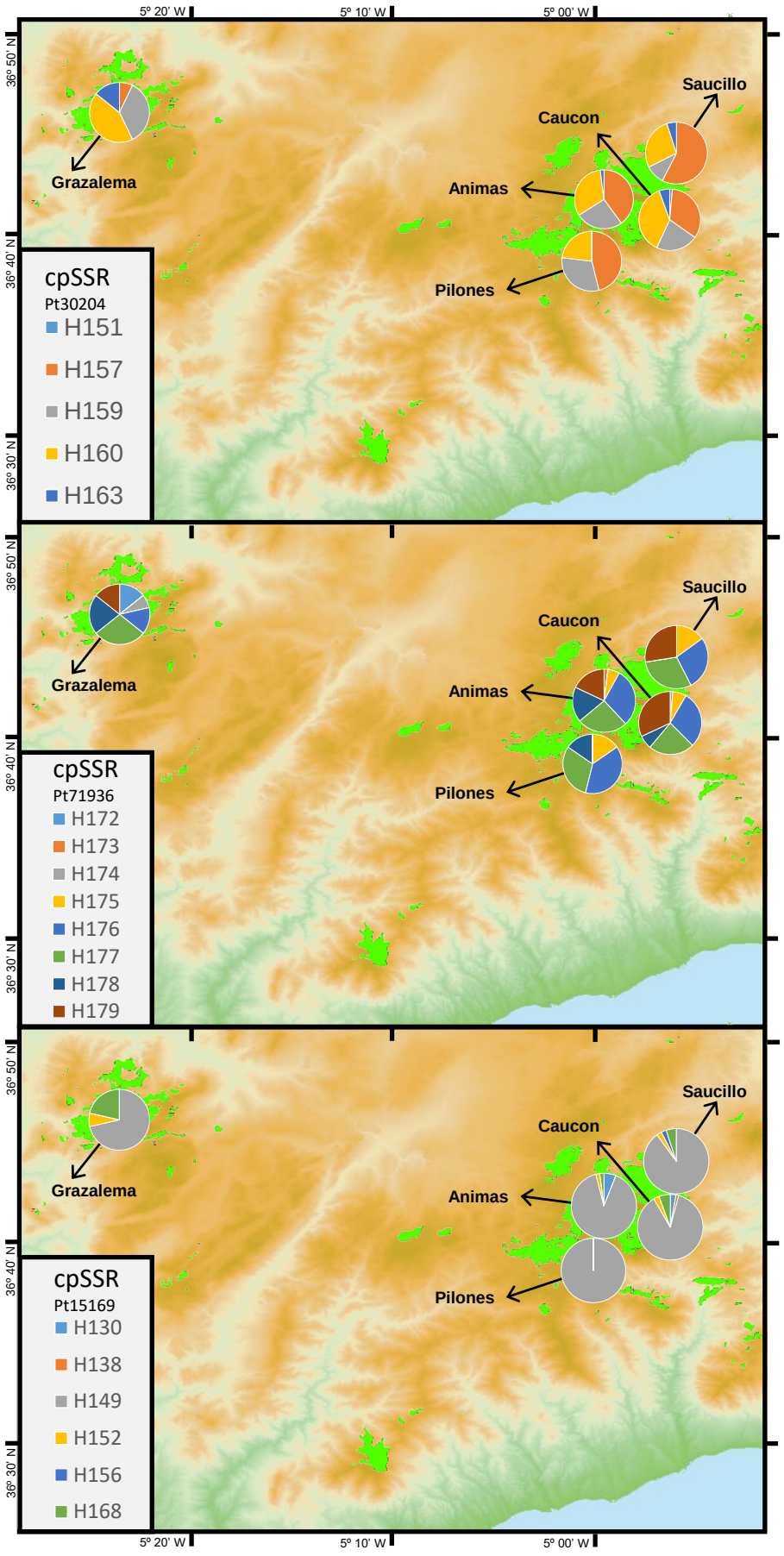
	nSSR						cpSSR			ISSR		
Code	NPA	Ne	Ar (gene copies)	Ho	He	Fis (p-value)	NPA	Ne	h	NPA	Ne	He
S	1.13	3.032	2.872 (6)	0.431	0.589	0.265 (<0.001)	0.333	2.469	0.514	0	1.045	0.029
SL		2.966	3.890 (12)	0.471	0.603	0.225 (<0.001)		2.15	0.431		1.037	0.024
SM		2.707	3.495 (12)	0.403	0.549	0.264 (<0.001)		2.421	0.575		1.051	0.03
SH		2.797	3.886 (12)	0.402	0.581	0.311 (<0.001)		2.094	0.524		1.037	0.024
SY		2.585	2.698 (6)	0.369	0.558	0.336 (<0.001)		2.459	0.545		1.043	0.026
SA		2.954	2.878 (6)	0.438	0.568	0.238 (<0.001)		2.116	0.53		1.044	0.026
SO		3.227	3.082 (6)	0.521	0.636	0.188 (<0.001)		2.069	0.449		1.041	0.026
C	0.38	3.126	2.896 (6)	0.564	0.613	0.08 (0.04)	0.667	2.847	0.565	1	1.079	0.047
CL		2.951	4.791 (26)	0.581	0.617	0.060 (0.157)		2.317	0.465		1.072	0.041
CM		3.109	4.946 (26)	0.568	0.614	0.077 (0.03)		3.223	0.63		1.082	0.049
CH		2.581	4 (26)	0.5	0.572	0.131 (0.04)		2.305	0.474		1.067	0.039
CY		2.981	5.375 (46)	0.527	0.598	0.121 (0.014)		2.575	0.511		1.064	0.04
CA		3.165	5.463 (46)	0.547	0.618	0.118 (0.009)		2.508	0.507		1.075	0.043
CO		3.06	5.75 (46)	0.603	0.625	0.036 (0.271)		3.022	0.636		1.084	0.049
A	0.63	3.11	2.830 (6)	0.594	0.612	0.028 (0.236)	0.333	2.89	0.554	0	1.082	0.049
AY		3.019	4.148 (18)	0.644	0.627	0.028 (0.714)		2.737	0.561		1.087	0.051
AA		2.845	4.146 (18)	0.593	0.633	0.053 (0.297)		2.606	0.496		1.043	0.025
AO		2.969	3.748 (18)	0.544	0.574	0.054 (0.202)		2.812	0.592		1.079	0.045
G	0	2.563	2.590 (6)	0.598	0.546	-0.100 (0.913)	0.333	3.303	0.685	0	1.812	0.048
GL		2.283	2.5 (4)	0.688	0.604	-0.222 (0.999)		2	1		1.069	0.037
GM		2.288	2.12 (4)	0.604	0.504	-0.224 (0.978)		3.257	0.822		1.055	0.031
GH		2.586	2.303 (4)	0.563	0.6	0.069 (0.385)		1.933	0.444		1.073	0.04
P	0	3.035	2.750 (6)	0.481	0.631	0.245 (<0.001)	0	2.406	0.487	0	1.061	0.037
Total		2.825	2.788 (6)	0.528	0.596	0.120 (<0.001)		3.722	0.523		1.058	0.035

3.3. Spatial genetic structure and demographic history

Overall, the data showed no significant differences for among population genetic differentiation. Hence, nSSR markers showed *Ar* for a standardized sample size of 6 ranging from 2.590 in Grazalema to 2.896 in Caucon (Table 2). *He* values ranged from 0.546 in Grazalema to 0.631 in Pilones. On the other hand, *FIS* was very high and statistically significant in Saucillo and Pilones populations (FIS=0.265, P<0.001 and FIS=0.245,

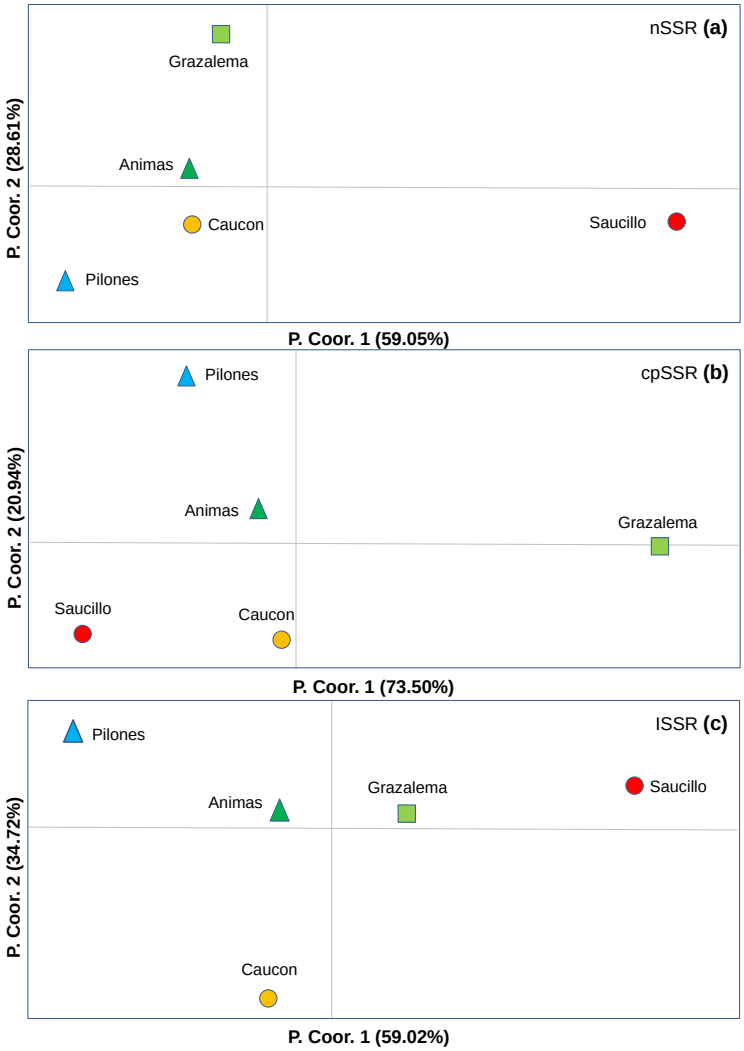
302 p<0.001 respectively) and moderate but statistically significant in Caucon (FIS=0.080, p=0.004). However,
303 Animas and Grazalema populations showed a very low value that was not significantly different from 0 (FIS =
304 0.028, P = 0.236 and FIS=-0.100, P=0.913, respectively). For cpSSR markers, *h* ranged from 0.487-0.685. For
305 ISSR markers, *He* values were between 0.029-0.049 (Table 2). In addition, the Grazalema population showed
306 the widest variety of haplotypes and the most equally distributed (Figure 2). Student's t test for unequal
307 variances showed no statistically significant differences, nor between Sierra de las Nieves and Grazalema for
308 the analysed neutral markers, neither among the different studied populations (p>0.05 in all cases), with the
309 exception of FIS between Grazalema and Sierra de las Nieves (p = 0.001), as well as among Sierra de las Nieves
310 populations (P<0.05), indicating that genetic diversity is very similar in all populations. Regarding genetic
311 diversity among populations, low to moderate differences were observed based on all three neutral markers
312 (Supplementary material, Tables S2-S4).

313 Hierarchical AMOVA for nSSR markers based on Fst and Rst showed a 7% and 9% of the total genetic
314 variance due to differences among populations, a 10% and 6% among individuals within populations and 83 %
315 and 85% within individuals, respectively. For cpSSR and ISSR, hierarchical AMOVA based on PhiPT showed
316 a 5% and 12% of the total genetic variance due to differences among populations and a 95% and 88% due to
317 differences within populations, respectively. The genetic differences among populations were statistically
318 significant in all cases (nSSR: Fst, p=0.001 and Rst, p=0.001; cpSSR: PhiPT, p=0.04; ISSR, PhiPT, p=0.001).
319 Moreover, pairwise Fst and Nei distances were statistically significant based on all analysed markers and
320 showed the highest differences in Grazalema, based on chloroplast markers (cpSSR) and Saucillo, based on
321 nuclear markers (nSSR and ISSR), congruently with the geographical distribution of populations, as Saucillo
322 represents the westernmost population and Grazalema the easternmost one (Figures 1 and 2). For nSSR,
323 pairwise Fst and Nei were congruent in their results, and all pairwise Fst differences were significantly larger
324 than 0 (p<0.05). For cpSSR, Nei distances ranged from 0.224 to 0.107 and for ISSR, from 0.0249 to 0.00597.
325 PCoA results (Figure 3; Electronic Supplementary material, Figure S1) were consistent with the previous
326 analysis, showing differentiation in Saucillo, based on nuclear markers (nSSR and ISSR), and Grazalema, based
327 on chloroplast markers (cpSSR; Figures 2 and 3).



328

329 **Figure 2.** Distribution pattern of Pt30204, Pt71936, and Pt15169 cpSSR haplotypes.



330

331 **Figure 3.** PCoA analyses based on pairwise Nei's standard genetic distances sorted by populations:
332 nSSR (a), cpSSR (b), and ISSR (c).

333 STRUCTURE analyses (Electronic Supplementary material, Figure S2) separated some of the *A. pinsapo*
334 populations based on nuclear markers (nSSR, ISSR). Based on nSSR, Saucillo was separated, in agreement
335 with Fst and Nei distances (Table 2) and PCoA results (Figure 3). ISSR markers also yielded a separation
336 among the different populations. However, STRUCTURE analyses based on cpSSR did not separate the
337 different studied populations. Isolation by distance (IBD) assessed over all populations did not shown
338 statistically significant result based on nuclear markers (Mantel test, nSSR, p=0.412; ISSR, p=0.284). However,
339 it was weak but significant, based on chloroplast markers (cpSSR, p=0.044) yet with the RMA regression
340 explaining only 1% of the variance in the whole distribution area (R^2=0.0105). The BOTTLENECK analysis
341 based on nSSR showed evidence of significant heterozygote excess (recent decline) in Saucillo (p = 0.02),
342 Caucon (p = 0.037), Pilones (p = 0.019) and Grazalema populations (p = 0.009).

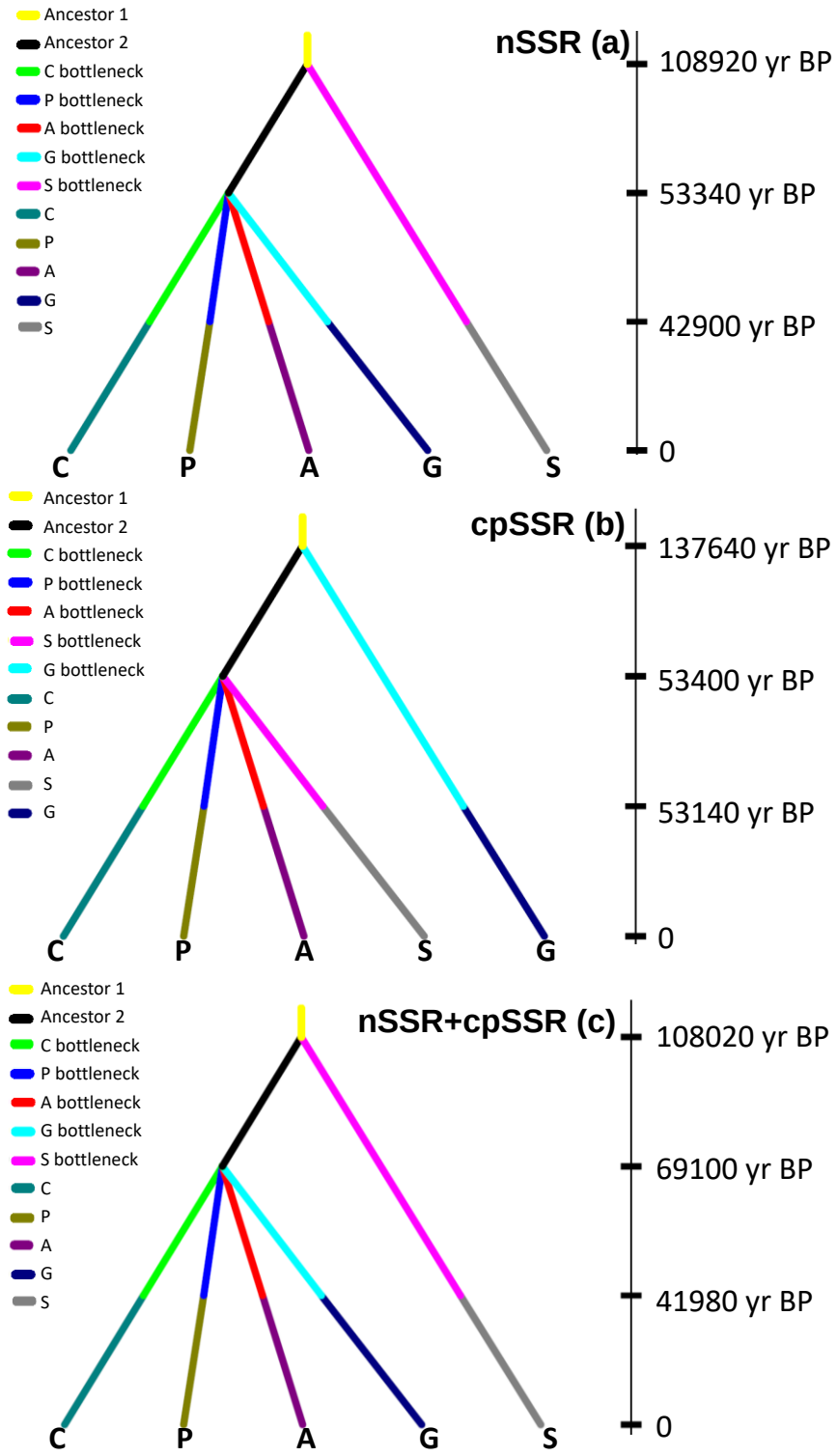


Figure 4. Likelihood scenarios for differentiation among the studied *Abies pinsapo* populations based on nSSR markers (a); cpSSR markers (b); and using both, nSSR and cpSSR markers (c); t(i) indicates time scale measured in generations; the segments indicates the effective population sizes prior and after simulated bottlenecks. S, Saucillo; C, Caucon; A, Animas; P, Pilones; and G, Grazalema. See also geographic locations in Figure 1.

DIYABC 2.1.0 results (Figure 4) showed the following evolutionary scenarios: (Figure 4a) based on nuclear markers, Saucillo population diverged from an ancient ancestral population and then, the rest of populations split at the same time with a recent bottleneck in all populations. (Figure 4b) based on chloroplast markers, Grazalema population diverged from an ancient ancestral population and then, the rest of population split at the same time with also a recent bottleneck in all populations. These results were consistent with the previously showed above, which pointed to Grazalema as the most different population based on chloroplast markers and Saucillo based on nuclear markers, and also with the BOTTLENECK results, which pointed to the existence of recent bottlenecks in all populations with the exception of Animas. However, Animas also showed evidence of a recent bottleneck based on DIYABC results. The posterior probabilities of these scenarios were 0.709 (95% CI = 0.3112–1.0000) and 0.899 (95% CI = 0.8414–0.9532) for direct estimation and logistic regression respectively, based on nSSR; and 0.1102 (95% CI = 0.0000–0.3846) and 0.6956 (95% CI = 0.6000–0.8459) based on cpSSR.

Finally, (Figure 4c) using nSSR and cpSSR together to carry out the analysis, the most probable scenario was Saucillo divergence from an ancient population in the first place followed by the rest of populations splitting at the same time, with a recent bottleneck in all of them (Figure 4c). The posterior probabilities of this scenario were 0.415 (95% CI = 0.00325-0.84592) and 0.516 (95% CI = 0.3193-0.7130). The effective population size based on nSSR was lower in Saucillo and Caucon and similar among the other populations. However, cpSSR predicted the highest effective population size for Animas (Electronic Supplementary material, Tables S5 and S6).

3.4. Genetic differentiation among spatial and temporal cohorts

Individuals belonging to high, middle and low elevation, as well as young, adult and old trees, showed non-statistically significant differences for the studied molecular markers ($P > 0.05$ in all cases; Student's t tests), indicating that there are no differences in terms of neutral genetic diversity related to altitudinal gradients or age-related cohorts. In addition, the number of migrants (Nm) based on the Fst of nSSR from different elevations showed high values in Saucillo, Caucon and Grazalema, ranging from 3.32 to 20.5. Hierarchical AMOVA analysis did not showed any significant differences related to elevation and cohorts, based on chloroplast markers but some significant differences were found based on nuclear markers: Saucillo (Fst=0.02, p=0.02) and Caucon (Fst=0.01, p=0.04) by elevation based on nSSR (Fst=0.091, p=0.005), and Grazalema by elevation (PhiPT=0.16, p=0.003) together with Saucillo and Animas by age (PhiPT= 0.22, p=0.001 and 0.16, p=0.002, respectively) based on ISSR.

4. Discussion

4.1. Genetic diversity of neutral molecular markers.

We hypothesised a low genetic diversity for *A. pinsapo*, according to the long-term isolation and relict character of this Mediterranean fir (Hampe and Petit 2005; Linares, 2011; Hampe and Jump 2011). However, the patterns obtained here were congruent among markers, supporting a relatively high within-population genetic diversity. Hence, nSSR markers revealed that *A. pinsapo* holds a relatively high genetic diversity (*He* = 0.596±0.034) and allelic richness (*Ar*=2.79). High molecular diversity has been previously reported in this (Dering et al., 2014; Sanchez-Robles et al., 2014) and other relict conifers, such as Serbian spruce (*Picea omorika* (Panč.) Purk.; Aleksić and Geburek 2013) and Chihuahua spruce (*Picea chihuahuana* Martinez; Ledig et al., 1997; Jaramillo-Correa et al. 2006; Wehenkel and Saenz-Romero 2012). These findings suggest that the observed high levels of genetic diversity of several relict conifers rely on frequent past admixture events of genetically differentiated populations (Alba-Sánchez et al., 2010; Eliades et al., 2011; Linares 2011; Aleksić and Geburek 2013), supporting a different way of retention of genetic variants, compared to other conifers with broad ranges, whose molecular diversity seems to be maintained by large effective populations sizes (Vendramin et al., 1999; Parducci et al., 2001; Liepelt et al., 2010).

Secondly, we also hypothesised the existence of inbreeding and recent bottlenecks, driven by long-term isolation of the remnant *A. pinsapo* populations. The inbreeding index obtained here (*Fis*=0.150) was higher than those obtained previously in *A. pinsapo* (Dering et al., 2014) and other relict circum-mediterranean fir, such as *A. cilicica* (Awad et al., 2014). However, the high inbreeding index showed by some populations (specifically, Caucon, Saucillo and Pilones), may be related to our sample design since Caucon and Saucillo populations (Figure 1) were sampled by selecting old individuals and their closest mature trees and saplings. As a consequence, neighbouring trees might be related, likely affecting the *Fis* estimates. Indeed, Grazalema and Animas populations (Figure 1), which were randomly sampled, did not showed significant inbreeding. Nonetheless, the inbreeding of these *A. pinsapo* populations might be also related to land-use legacies (Reed and Frankham 2003; Kremer et al., 2012) since Caucon and Saucillo populations were subjected over centuries to intensive human perturbations, such logging and grazing, until these forests were declared as protected areas (Linares et al., 2009; Lechuga et al., 2017). Although Grazalema and Animas populations were also subjected to logging and grazing, the first belonged to private owners, while the second belonged to a municipality where logging and grazing were less intense (Linares et al., 2009). Finally, Pilones population represents one of the current treeline ecotones of *A. pinsapo*, which seems to be expanding upward as a consequence of both global

409 warming and land-use changes (Lechuga et al., 2019). Thus, the significant inbreeding index obtained in these
410 randomly sampled individuals could be related to their recent expanding dynamics from a limited number of
411 leading-edge individuals (Hampe and Petit 2005), although the effects of a limited sample size (n=13) and the
412 high frequency of null alleles (0.101; Table S1) may also play a role here.

413 *4.2. Spatial genetic structure and demographic history.*

414 Spatial differentiation regarding the among-populations genetic structure should be expected in relict tree
415 species (Clark et al., 2000; Eliades et al., 2011; Hampe and Jump 2011). Accordingly, geographic location was
416 hypothesized here to affect genetic diversity, both within and among *A. pinsapo* populations. The studied *A.*
417 *pinsapo* populations revealed an overall low genetic differentiation and low spatial genetic structure. Despite
418 the low spatial genetic structure inferred here, the genetic differentiation was statistically significant. Besides,
419 the inferred demographic history supports a bottleneck effect experienced in all the studied populations. Indeed,
420 most circum-mediterranean relict species may have suffered a genetic bottleneck at some point in their
421 evolutionary history, resulting in a dramatic decrease in genetic diversity (Fady-Welteren, 2005; Fady and
422 Conord 2010; Linares, 2011).

423 The earlier differentiation among the studied populations occupies the time between ca. 140 and ca. 100
424 thousand years before present (kyr BP). This period is of particular interest with regard to orbital parameters,
425 contrasted vegetation changes and climatic conditions (Cheddadi et al., 1998). Although estimates of time
426 frames affecting the studied *A. pinsapo* populations must be taken with caution, this former differentiation
427 among the studied populations of *A. pinsapo* might be related to the major cooling episode that occurred after
428 the Last Interglacial (Combourieu-Nebout et al., 2002; Fletcher and Sanchez Goñi 2008). Further bottlenecks,
429 inferred from ~70–40 kyr BP in all the studied populations, may be related to abrupt climate changes, such as
430 the Dansgaard-Oeschger (DO) and the Heinrich (H) events, occurring during the last glacial cycle and the
431 Holocene (Heinrich, 1988; Dansgaard et al., 1993). The periods between ~115–100 kyr BP and ~75–45 kyr BP
432 have been related to regional-scale dry conditions in the west Mediterranean, based upon low groundwater
433 carbonate deposition and pollen-based palaeoclimate reconstructions (Cheddadi et al., 2005; Camuera et al.,
434 2019). Then, it is assumed that steppe-like vegetation predominated during these cold-dry events of the last
435 glacial stage such that the estimated *A. pinsapo* genetic bottlenecks might correspond to dramatic decreases in
436 genetic diversity linked to DO oscillations and H events.

437 The Quaternary genetic and demographic changes of other relict conifer, such as the cold-adapted spruce
438 *P. omorika*, suggest that scattered populations were subjected to long-term genetic isolation and related genetic

drift effects, that continuously increased among-populations genetic distinctiveness during the last glacial and post-glacial (Aleksić and Geburek 2013). Nonetheless, due to their proximity to coastal glacial refugia, populations of *A. pinsapo* have likely experienced buffered climatic fluctuations during the last glacial termination (about 17.7–11.5 kyr BP; Linares, 2011). Indeed, while the glaciers were receding, periods of intense cold and dry climate have been recorded, such as the Younger Dryas (12.9-11.7 kyr BP), which was not inferred in any of our bottleneck modelling. In summary, the results obtained here, as well as those previously published (Dering et al., 2014), support that *A. pinsapo* has been sensitive to climatic changes occurring during the last glacial, as DO oscillations and H events, whilst the local-scale climate gradients might be ensured the persistence of remnant stands (Linares, 2011). The relatively stable Holocene climate has also experienced some intervals of rapid climate change that might have affected some *A. pinsapo* populations (Alba-Sánchez et al., 2010; Dering et al., 2014). Additionally, the increasing human-induced habitat fragmentation after the last glaciation likely limited the recolonization chance of remaining *A. pinsapo* populations (Alba-Sánchez et al., 2019). Long-term logging and overgrazing activities have been related to declining genetic variation, as a result of severe habitat fragmentation and temporal fluctuations in demographic parameters (Clark et al., 2000; Wehenkel and Saenz-Romero 2012). Here, using paternally inherited cpSSR markers, we obtained the highest effective population size for Animas (Table S4), according to the currently better-preserved old-growth *A. pinsapo* forest (Alba-Sánchez et al., 2019).

The presence of two main *A. pinsapo* forests in South Spain (Figure 1), surrounded by several scattered individuals and isolated small stands, suggests a wider former distribution (Linares 2011; Dering et al., 2014). Hence, the weak geographic differentiation patterns obtained here might be explained by ensuing pollen-mediated gene flow, as few migrants per generation are required to prevent divergence between subpopulations for neutral markers (Clark et al., 2000; Petit et al., 2005; Chapuis and Estoup 2007; Kremer et al., 2012). However, the restricted pollen dispersal of *A. pinsapo*, estimated as less than 3 km (Arista and Talavera 1994b; Alba-Sánchez et al., 2010), and the low value of among-populations Nm obtained (1.69) contrast with this hypothesis. The higher genetic differentiation between the populations of Grazalema and Sierra de las Nieves based on chloroplast markers is consistent with their geographical distribution and agrees with some relationships previously obtained between genetic and geographic distance also using cpSSR data (Terrab et al., 2007). Hence, our results support that limited gene flow by pollen and almost complete lack of seed flow may prevent genetic connectivity and enhance genetic differentiation among populations distant by a few kilometres (Kremer et al., 2012; Aleksić and Geburek 2013).

We found that Grazalema contains a higher number of haplotypes for all three chloroplast markers, compared to Sierra de las Nieves populations (Figure 2), suggesting the legacy of contrasting history, while nuclear markers did not support this differentiation. This decoupled population genetic structure, based on different-inherited DNA markers has been related to increasing genetic differentiation among different ancestral populations (Clark et al., 2000; Petit et al., 2005; Jaramillo-Correa et al., 2006). Thus, comparison between the genetic diversity of maternally inherited mitochondrial and paternally inherited chloroplast DNA markers in the relict spruce *P. chihuahuana* showed higher cpDNA diversity, while these cpDNA markers showed low population differentiation (Jaramillo-Correa et al., 2006). Our PCoA analyses based on Nei's pairwise genetic distances revealed the highest differences in Grazalema, based on chloroplast markers (cpSSR) and Saucillo, based on nuclear markers (both, nSSR and ISSR), which represents the westernmost and easternmost populations, respectively (Figure 1).

Although these chloroplast and nuclear diversity estimates do not show genetic differentiation for quantitative traits of adaptive relevance, this differentiation between the westernmost and easternmost populations might be related to local climate gradients (Linares et al., 2011). Most of the annual rainfall, carried by low pressure systems coming from Atlantic depressions, falls on the western part of the study area and decreases toward the eastern part (Linares et al., 2011) such that high mean precipitation values occur in the westernmost population of Grazalema, as compared to the easternmost population of Saucillo. This longitudinal differentiation was also reflected in the demographic history inferred by DIYABC using nSSR and cpSSR markers (Figure 4) indicating that potential adaptive divergence of easternmost and westernmost populations should be subject to further research. Similar spatial differentiation has been suggested for *A. cilicica* in Lebanon, where it grows as remnant populations (Awad et al., 2014), although, contrasting to this research, we did not detect significant genetic differentiation related to elevation.

The different cohorts studied here (old, mature and young trees) did not show statistically significant differences in genetic diversity. Thus we conclude that, at least under the time scale investigated here, the populations are not undergoing genetic erosion. Studies reporting significant genetic erosion among cohorts of trees species are very scarce. For instance, the genetic diversity obtained across diameter classes, used as a surrogate for age classes, of the relict spruce *P. chihuahuana* decreased significantly in only one very small population (Wehenkel and Saenz-Romero 2012). Thus, the current distribution *A. pinsapo* in south Spain appears to be the result of long-term range retraction and local persistence as marginal populations (Terrab et al., 2007; Linares 2011; Dering et al., 2014; Sanchez-Robles et al., 2014).

4.3. Concluding remarks and conservation insights

Our results support relatively high levels of genetic diversity in this species. Conservation actions are generally based on adaptive genetic variation, which often does not match with neutral molecular variation. It must be stressed that most variation among the *A. pinsapo* populations, reported here and by previous studies, might be likely caused by genetic drift. While drift is not expected to routinely affect fitness, it can lead to the fixation of some alleles and the loss of others (Hampe and Petit 2005; Hampe and Jump 2011). Under a drier and warmed climate, the genetic diversity observed in this relic and drought-sensitive fir would be subjected to selective pressure, no matter if this genetic diversity was essentially determined by random genetic drift. Hence, the coming patterns will likely differs from the neutral expectations and they may provide unexpected adaptive consequences (Kuparinen et al., 2010; Alberto et al., 2013). Knowledge on adaptive genetic variation in *A. pinsapo* is still lacking, while significant phenotypic plasticity regarding carbon and water balance responses to local climate suggests putative adaptive capacity in this relict fir (Lechuga et al., 2019 and references therein). Hence, further research is necessary to assess the putative loss of evolutionary potential in these stands as well as to identify divergence patterns of adaptive relevance.

The genetic differentiation of some populations, particularly Saucillo and Grazalema, may guide further research focused on adaptive evolutionary processes, such as epigenetic mechanisms or phenotypic traits related to drought tolerance (Neale and Wheeler 2019). Such research should also include the conservation status and management of the closely related North-African populations of *A. marocana* and *A. tazaotana* (Terrab et al., 2007; Jaramillo-Correa et al., 2010; Dering et al., 2014; Sanchez-Robles et al., 2014). Conservation efforts should focus on reducing the probability of stochastic events, such as fires together with preventing further habitat loss due to human impacts or climate change, while ex-situ conservation of genetic resources or assisted migration would be also valuable given the limited migration potential of the species due to topographic and landscape constraints. Furthermore, managing stand structure to reduce competition provides a promising strategy to reduce climate change risks on some drought-sensitive tree species (Lechuga et al 2017, 2019). Weak competitive ability has already been stated, for instance, in relict yew (*Taxus baccata* L.) populations (Iszkuło et al 2012), while removal of competing vegetation has been recommended as adaptive management in other relict conifers (Wehenkel and Saenz-Romero 2012).

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Jump conducted the statistical analyses results discussion. All authors contributed to the final writing of the manuscript.

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Supplementary material

Appendix 1. *DNA Extraction, Microsatellite and Inter-Microsatellite Genotyping*

Total genomic DNA was successfully extracted and purified from 100 mg of leaves per sample using the QIAGEN DNeasy plant mini kit according to manufacturer's protocol (Pérez-González et al., 2018). Quality and quantity of the DNA extraction was measured by means of 1% agarose gel and Nanodrop, respectively. We used 8 nuclear microsatellites to perform genomic DNA amplification: NFF2, NFF3, NFH15, NFH3 and NFF7 developed for *A. nordmanniana* Stev. (Hansen et al., 2005); and Pin8, Pin20 and Pin48 developed for *A. pinsapo* (Sánchez-Robles et al., 2012). In addition, we amplified 3 chloroplastic microsatellites: Pt30204, Pt71936 and Pt15169 developed for *Pinus thunbergii* (Vendramin et al., 1996). Microsatellite selection was done based on previous studies that prove that they yield enough polymorphic bands in other species phylogenetically related with *A. pinsapo*. 4 fluorescent dyes were used to label forward primers on their 5' end: FAM (blue), VIC (green), PET (yellow) or NED (red) (Eurofins MWG Operon). Then, all individuals were amplified by polymerase chain reaction (PCR). The PCR mix contained 5 microlitres of DNA AmpliTools Master Mix 2x (Biotools), 1 microlitres of each primer, fordward and reverse (5 mM), 1 microlitre of fluorescent dye, 0.5 microlitre of template DNA (30 ng) and 1.5 microlitres of autoclaved miliQ purified water to obtain a total volume of 10 microlitres for each sample. The thermal cycling consisted of an initial denaturation step at 94ºC for 3 min, 3-step cycling repeated 35 times and consisting of denaturation at 94º for 1 minute, annealing at 56º for 1 minute and extension at 72º for 80 seconds; and a final extension step at 72º for 8 minutes (Pérez-González et al., 2018).

ABI 3730XL automated sequencer (Applied Biosystems) with the GeneScan™ - 500 LIZ™ size standard (Applied Biosystems) was used to perform a capillary electrophoresis with the obtained PCR products. Allelic binning and scoring of genotypes were carried out manually by two different people using the software GeneMapper 4.1 (Applied Biosystems) and compared to get the final data set, with the objective of reducing the possibilities of genotyping mistakes related to automated or arbitrary decisions in allelic binning (Amos et al., 2007). 19 ISSR primers from the University of British Columbia, Canada (UBC) were tested in two individuals to select those that yield more polymorphic bands. They were amplified by polymerase chain reaction (PCR). The PCR mix contained 5 microlitres of DNA AmpliTool Master Mix 2x (Biotools), 2 microlitres of primer (5 mM), 0.5 microlitres of template DNA (30 ng) and 2.5 microlitres of autoclaved miliQ purified water to give a total volume of 10 microlitres for each sample. The thermal cycling consisted of an initial denaturation step at

94ºC for 5 min, 3-step cycling repeated 35 times and consisting of denaturation at 94º for 30 seconds, annealing at 52º for 45 seconds and extension at 72º for 2 minutes; and a final extension step at 72º for 6 minutes.

The PCR products were analysed using a multicapillary electrophoresis system with a modified AL420 method file (QIAxcel DNA High Resolution Kit). Two replicates of the PCR products were made to evaluate the consistency of the bands obtained. ISSR band outputs were counted automatically using the QIAxcel Bio Calculator with thresholds for similarity set a baseline filter = 100 rfu, threshold = 15 %, minimum distance = 2.00 bp. Each sample profile was tested visually to eliminate miscalled or poorly identified peaks. Then, those bands that were not found in both replicates of each individual were removed. Thus, we ensure the repeatability of the bands. The QIAxcel Bio Calculator was used to produce a presence/absence binary score for each sample. For each primer, amplified fragments with the same molecular weight (bp) were documented as present (1) or absent (0). We used the obtained binary matrix in the further analyses. We accepted that each band showed one Mendelian locus with two alleles, the 'dominant' or visible alleles and the 'recessive' or null alleles. We also accepted that alleles from different loci do not migrate at the same position.

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787 **Table S1**. Null alleles frequency estimated by Expectation Maximization (EM) algorithm in nSSR loci.
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		Null alleles estimated frequency
Locus	NFF2	0.052
	NFF3	0.044
	NFH15	0.024
	NFH3	0.044
	NFF7	0.044
	Pin8	0.034
	Pin20	0.074
	Pin48	0.084
Population	S	0.086
	C	0.031
	A	0.024
	G	0.013
	P	0.101

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793 **Table S2.** Hierarchical AMOVA based on ISSR markers for different levels of analysis (among
794 populations, among elevation cohorts and among age cohorts). *Statistically significant p-values.
795

ISSR	Level of analysis	df	SS	MS	Est. Var.	%	PhiTP	P-value
5 pops	Among Pops	5	256.334	51.267	1.386	12%	0.120	0.001*
	Within Pops	194	1969.331	10.151	10.151	88%		
	Total	199	2225.665		11.537	100%		
Saucillo by elevation	Among Pops	2	1.149	0.574	0.000	0%	-0.048	0.957
	Within Pops	39	55.604	1.426	1.426	100%		
	Total	41	56.753		1.426	100%		
Caucon by elevation	Among Pops	2	2.354	1.177	0.000	0%	-0.020	0.894
	Within Pops	69	141.877	2.056	2.056	100%		
	Total	71	144.231		2.056	100%		
Grazalema by elevation	Among Pops	2	7.151	3.576	0.316	16%	0.159	0.003*
	Within Pops	15	25.167	1.678	1.678	84%		
	Total	17	32.318		1.994	100%		
Saucillo by age	Among Pops	3	154.786	51.595	3.743	22%	0.222	0.002*
	Within Pops	38	497.500	13.092	13.092	78%		
	Total	41	652.286		16.835	100%		
Caucon by age	Among Pops	2	4.787	2.394	0.016	1%	0.008	0.248
	Within Pops	69	139.498	2.022	2.022	99%		
	Total	71	144.285		2.037	100%		
Animas by age	Among Pops	2	17.314	8.657	0.400	16%	0.162	0.001*
	Within Pops	48	99.118	2.065	2.065	84%		
	Total	50	116.433		2.465	100%		

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798 **Table S3.** Hierarchical AMOVA based on cpSSR markers for different levels of analysis (among
799 populations, among elevation cohorts and among age cohorts) *Statistically significant p-values.
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cpSSR	Level of analysis	df	SS	MS	Est. Var.	%	PhiPT	P-value
5 pops	Among Pops	4	335.228	83.807	1.655	5%	0.041	0.038*
	Within Pops	184	6186.084	33.620	33.620	95%		
	Total	188	6521.312		35.075	100%		
Saucillo by elevation	Among Pops	2	73.424	36.712	1.104	5%	0.046	0.143
	Within Pops	37	844.726	22.830	22.830	95%		
	Total	39	918.150		23.934	100%		
Caucon by elevation	Among Pops	2	6.429	3.215	0.000	0%	-0.045	0.962
	Within Pops	67	2310.328	34.483	34.483	100%		
	Total	69	2316.757		34.483	100%		
Grazalema by elevation	Among Pops	2	230.667	115.333	11.992	16%	0.158	0.167
	Within Pops	11	703.333	63.939	63.939	84%		
	Total	13	934.000		75.931	100%		
Saucillo by age	Among Pops	3	40.150	13.383	0.000	0%	-0.049	0.735
	Within Pops	36	878.000	24.389	24.389	100%		
	Total	39	918.150		24.389	100%		
Caucon by age	Among Pops	2	40.014	20.007	0.000	0%	-0.018	0.613
	Within Pops	67	2276.743	33.981	33.981	100%		
	Total	69	2316.757		33.981	100%		
Animas by age	Among Pops	2	11.320	5.660	0.000	0%	-0.055	0.948
	Within Pops	47	1609.100	34.236	34.236	100%		
	Total	49	1620.420		34.236	100%		

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803 **Table S4.** Hierarchical AMOVA based on nSSR markers for different levels of analysis (among

804 populations, among elevation cohorts and among age cohorts).

nSSR	Level of analysis	df	SS	MS	Est. Var.	%	Fst	P-value
5 pops	Among Pops	5	61.017	12.203	0.170	7%	0.065	0.001*
	Among Indiv	185	495.166	2.677	0.257	10%		
	Within Indiv	191	413.036	2.162	2.162	84%		
	Total	381	969.220		2.589	100%		
Saucillo by elevation	Among Pops	2	8.321	4.161	0.049	2%	0.020	0.017*
	Among Indiv	37	108.673	2.937	0.603	25%		
	Within Indiv	40	69.253	1.731	1.731	73%		
	Total	79	186.247		2.383	100%		
Caucon by elevation	Among Pops	2	7.701	3.850	0.029	1%	0.012	0.038*
	Among Indiv	67	176.421	2.633	0.199	8%		
	Within Indiv	70	156.500	2.236	2.236	91%		
	Total	139	340.621		2.463	100%		
Grazalema by elevation	Among Pops	2	3.095	1.548	0.000	0%	-0.026	0.742
	Among Indiv	11	22.333	2.030	0.000	0%		
	Within Indiv	14	33.500	2.393	2.393	100%		
	Total	27	58.929		2.393	100%		
Saucillo by age	Among Pops	3	8.294	2.765	0.000	0%	-0.006	0.684
	Among Indiv	36	108.640	3.018	0.643	27%		
	Within Indiv	40	69.269	1.732	1.732	73%		
	Total	79	186.204		2.375	100%		
Caucon by age	Among Pops	2	4.154	2.077	0.000	0%	-0.005	0.871
	Among Indiv	67	179.967	2.686	0.225	9%		
	Within Indiv	70	156.500	2.236	2.236	91%		
	Total	139	340.621		2.461	100%		
Animas by age	Among Pops	2	7.110	3.555	0.034	1%	0.014	0.065
	Among Indiv	47	116.610	2.481	0.053	2%		
	Within Indiv	50	118.791	2.376	2.376	96%		
	Total	99	242.511		2.462	100%		

805

Appendix 2. Evolutionary scenarios tested with DIYABC 2.1.0.

Seven evolutionary scenarios were tested with DIYABC 2.1.0 based on the results obtained with the different parameters used to test genetic differentiation among populations and assuming hypothetical divergence times (t1, t2, … t_n): (i) Grazalema and Sierra de las Nieves populations diverged from an ancestral population at t1, followed by Saucillo at t2 and then the rest of the studied populations (Caucon, Animas and Pilones) diverged simultaneously at t3; (ii) Saucillo and the rest of the populations diverged from an ancestral population at t1, followed by Grazalema at t2 and then the rest of the studied populations (Caucon, Animas and Pilones) diverged simultaneously at t3; (iii) Grazalema and the rest of the studied populations diverged from an ancestral population at t2, which split at the same time at time t3; (iv) Saucillo and the rest of the populations diverged from an ancestral population at t2, which split at the same time at time t3. These four scenarios were based on the fact that Saucillo and Grazalema constituted the most different populations based on the previous analyses. (v) Split from west: Grazalema and the rest of populations diverged from an ancestral population at t1, followed by Pilones at t2, Animas at t3, and Caucon and Saucillo at t4; (vi) Split from east: Saucillo and the rest of populations diverged from an ancestral population at t1, followed by Caucon at t2, Animas at t3, and Pilones and Grazalema at t4; (vii) split at the same time: all populations diverged from an ancestral population at time t1. All these scenarios were replicated to study the presence of bottlenecks, in order to test the results previously obtained by BOTTLENECK.

826 **Table S5**. Prior distributions of the parameters used in DIYABC analyses.

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Parameter	Minimum	Maximum
Effective population size	1	10000
Time scale in generations	1	10000
Mutation model		
Mean mutation rate	1×10^{-4}	1×10^{-3}
Individual locus mutation rate	1×10^{-5}	1×10^{-2}
Mean coefficient P	1×10^{-1}	3×10^{-1}
Individual locus coefficient P	1×10^{-2}	9×10^{-1}
Mean SNI rate	1×10^{-8}	1×10^{-4}
Individual locus SNI rate	1×10^{-9}	1×10^{-3}

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831 **Table S6**. Median values of effective population sizes estimated for the different ancestors,
832 populations prior to simulated bottlenecks and current populations (see Table 1 for abbreviations).
833 Divergence times (number of generations) were obtained by DIYABC using nSSR, cpSRR, and
834 both nSSR and cpSSR molecular markers together. Generation time was assumed 20 years for the
835 recalculation of historical time of divergence (Time BP 1, Time BP 2, Time BP 3, BP: years before
836 present).

837

	nSSR	cpSSR	nSSR + cpSSR
Ancestor 1	5804	7120	7157
Ancestor 1	4192	4032	5371
S	3120	2695	2437
C	1647	3282	3157
A	3518	4696	2913
P	3789	2219	3208
G	3852	1178	2959
S bottleneck	7915	7199	5984
C bottleneck	6150	6754	6882
A bottleneck	6490	6063	6914
P bottleneck	8263	6976	6401
G bottleneck	6166	6108	7352
Divergence 1	5446	6882	5401
Divergence 2	2677	2670	3455
Divergence 3	2145	2657	2099
Time BP 1	108920	137640	108020
Time BP 2	53340	53400	69100
Time BP 3	42900	53140	41980

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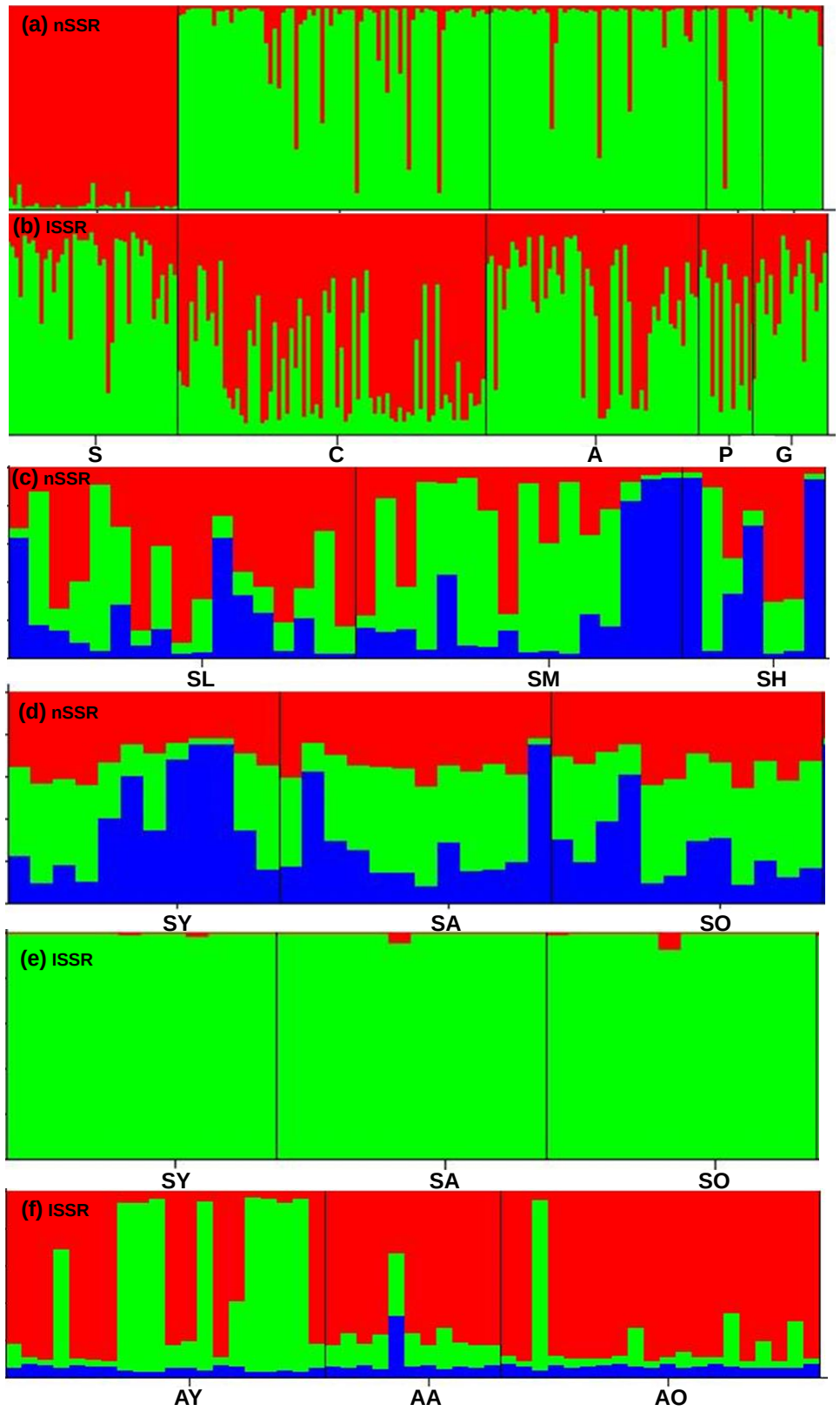
Figure S1. PCoA analyses based on pairwise Nei's standard genetic distances sorted by elevations and based on nSSR (a), and ISSR (b); and sorted by age cohorts and based on nSSR markers (c).



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845 **Figure S2.** Proportion of the membership coefficient for each individual in six *Abies pinsapo* forests based
846 on nSSR (a) and ISSR (b); Saucillo population based on nSSR sorted by elevation (c) and age (d), and based
847 on ISSR sorted by age (e); Animas population based on ISSR sorted by age (f). Inferred clusters used K = 2
848 (a, b and e) and K = 3 (c, d and f) in STRUCTURE analysis. Only results showing disconnected populations
849 are shown (see codes in Table 1).